MicroBioTest Protocol

Testing Pre-Saturated or Impregnated Towelettes for Tuberculocidal Effectiveness

Testing Facility
MicroBioTest
Division of Microbac Laboratories, Inc.
105 Carpenter Drive
Sterling, VA 20164

Prepared for
Professional Disposables International, Inc.
Two Nicepak Park
Orangeburg, NY 10962

December 5, 2014

MicroBioTest Protocol: 735.6.12.05.14

MicroBioTest Project No.: 735 . 156

OBJECTIVE:

This test is designed to substantiate tuberculocidal effectiveness for impregnated or pre-saturated towelettes, single or multiple uses, to be registered with the Environmental Protection Agency and Health Canada. The test incorporates appropriate aspects AOAC method 965.12 Tuberculocidal Activity of Disinfectants (2012). The test evaluates the effectiveness of products as disinfectants for contaminated surfaces. The test follows the "Germicidal Spray Products as Disinfectants" test as described in the Official Methods of Analysis, Eighteenth Edition, 2012, AOAC and the EPA Notice of Efficacy Requirements for Pre-Saturated or Impregnated Towelettes for Hard Surface Disinfection. This test also meets the EPA OCSPP 810.2000 and 810.2200 Product Performance Test Guidelines as applicable and follows the US. EPA OPP Microbiology Laboratory, SOP for Disinfectant Towelette Test against Mycobacterium bovis (BCG), SOP Number: MB-23-02, Date Revised: 03-05-13 where appropriate.

TESTING CONDITIONS:

A total of ten carriers per lot will be tested using two lots of a single test agent. The carriers, inoculated with *Mycobacterium bovis*, will be wiped following the procedure described in the protocol and held for the exposure time and at the temperature specified by the sponsor. The carriers will be cultured, incubated and observed for visible growth.

MATERIALS:

A. Test, control and reference agents will be supplied by the sponsor of the study (see last page).

- The identity, strength, purity, and composition, or other characteristics which will appropriately define the test, control, or reference agent shall be determined for each batch and shall be documented by the sponsor before its use in a study. Methods of synthesis, fabrication, or derivation of the test, control, or reference agent shall be documented and retained by the sponsor.

- When relevant to the conduct of the study the solubility of each test, control, or reference agent shall be determined by the sponsor before the experimental start date. The stability of the test, control, or reference agent shall be determined by the sponsor before the experimental start

date or concomitantly according to written standard operating procedures, which provide for periodic analysis of each batch.

The test agent will be tested as supplied by the sponsor unless directed otherwise. All operations performed on the test agent such as dilution or specialized storage conditions must be specified by the sponsor before initiation of testing.

The sponsor assures MicroBioTest, Division of Microbac Laboratories, Inc. (MicroBioTest) testing facility management that the test agent has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

MicroBioTest will retain all unused test agents for a period of one year after completion of the test, and then discard them in a manner that meets the approval of the safety officer.

B. Materials supplied by MicroBioTest including but not limited to:

1. Challenge organism, required by AOAC:
   Mycobacterium bovis (BCG). Organon Teknika, Corp.

2. Media and reagents:
   a. 0.85% NaCl containing 0.1% Polysorbate 80 (SS+).
   b. Middlebrook 7H11 agar.
   c. Kirchner medium.
   d. Middlebrook 7H9 broth.
   e. Phosphate Buffered Dilution Water (PBDW).
   f. Modified Proskauer-Beck Medium (MPB).
   g. Heat-inactivated fetal bovine serum (FBS), if required.
   h. Neutralizer: Heat-inactivated horse serum or other suitable solution

3. Laboratory equipment and supplies.

4. Laboratory equipment and supplies, including glass microscope slides (1" x 3" with a 1" x 1" surface for contamination and treatment)

TEST SYSTEM IDENTIFICATION:

All tube supports, baskets, or other culture-containing devices will be labeled with the following information: microorganism, test agent and project number.

EXPERIMENTAL DESIGN:

A. Preparation of inoculum:

From stock culture, tubes containing 20 mL of MPB will be inoculated by transferring one or two 1 µL loopfuls from 7H11 slants and incubated in a slanted position at 36±1C while remaining quiescent for 21±2 days.

Using a transfer loop, sufficient growth will be transferred from the surface of the 20 mL culture into a sterile tissue grinder. One mL of SS+ will be added and the culture will be macerated to break up large clumps. Nine mL of MPB will be added to the homogenized culture. The homogenized suspension will be transferred to a sterile tube and the culture will be allowed to settle for 10-15 minutes. The upper portion of the culture will be removed and transferred to a sterile flask, leaving behind any debris or clumps.

Using a spectrophotometer, the culture suspension will be standardized using MPB to achieve 20.0%±1% transmittance (T) at 650 nm.

If requested by the sponsor, HI FBS will be added to the culture to achieve 5% organic load.

B. Preparation of carriers:

The new carriers will be visually screened and discarded if visibly damaged (scratched, chipped or nicked). The carriers will be rinsed with 95% ethanol followed by a rinse with deionized water to remove oil and film on the slides. The carriers will be sterilized by placing in evaporating dishes matted with two pieces of filter paper, heating them in a hot air oven for two hours at 180C, cooling and storing them at room temperature until use.

C. Carrier inoculation:

Using a positive displacement pipette, a 0.01 mL (10 µL) aliquot of each culture will be transferred onto a one-square inch area on the sterile carriers (in Petri dishes) and immediately spread uniformly over the entire area with a sterile loop. Each dish will be covered promptly and the operation will be repeated for the rest of the carriers. Carriers will be dried for 30±2 minutes at 36±1C. The humidity level of the incubator during the drying phase required for the inoculated carriers will be monitored and reported.
Inoculated carriers will be used for testing within two hours of drying.

Note: The temperature and humidity level of the incubator during the drying of carriers will be monitored and reported.

D. Test material preparation:

Each lot of the test agent will be used as supplied by the sponsor in individual packages (one wipe per package).

The packaging for the test towelette will be massaged to distribute the liquid test agent into the towelette matrix before opening. The single towelette will be removed from the packaging.

Note: the towelettes (wipes) will be supplied in individual packets (one wipe per package). Each packet will be numbered and will include the sponsor-measured weight value. After opening, each packet will be weighed by MicroBioTest just prior to testing to verify that the weight matches the sponsor measure weight within +/- 2% range. If it does not, the weight will be documented and the wipe will not be used.

E. Test:

Note: The temperature and humidity level of the laboratory during the test phase will be monitored and reported.

Ten carriers will be evaluated. For each group of ten replicates, a single towelette will be used based on the following:

Each towelette will be folded in such a way that one towelette will be used to treat ten carriers. Each carrier (maintained in the Petri dish that was used for carrier inoculation) will be wiped. The area of the towelette used for wiping will be rotated to expose a new, unused surface for each carrier, allowing a maximum surface area of the towelette to be used over the course of the procedure.

Initially the towelette will be folded lengthwise twice and then folded five times inward beginning from the far end. Then the outside edges will be pulled upward to form a "U" shape and grasped preferably on one side with the thumb and on the other side with the index and middle finger.

Each contaminated carrier will be wiped using two complete horizontal strokes, with one right to left and back to right considered as one stroke; and then wiped using two complete vertical strokes, with one up to down and back to up considered one stroke for a total of four complete strokes. The used end will be flipped upward towards self, reoriented appropriately and then used to wipe the next carrier. The next three carriers will be wiped in a similar fashion - the used portion will be folded up-and-over each time. Once five carriers have been wiped, the second lengthwise fold will be unfolded and refolded in the opposite direction. The towelette will be refolded five times as before and the above procedure for wiping the first five carriers will be repeated for wiping the last five carriers. This process will be repeated until a total of ten carriers have been wiped with one towelette.

Each carrier will be held in a horizontal position for the exposure time as specified by the sponsor. After the contact period, the excess liquid will be allowed to drain from the carrier without touching the Petri dish or filter paper. Sequentially the carriers will be transferred into neutralizer tubes using sterile forceps within the ±5 seconds (or ±3 seconds) time limit and shaken thoroughly. For products with ≤1 minute contact time, the transfer will be made within ±3

seconds. The slide could touch both the interior sides of the Petri dish and the neutralizer tube during the transfer, but care will be taken to avoid this contact as much as possible.

Each tube containing carrier in neutralizer will be thoroughly shaken and transferred to a tube containing 20 mL of MPB broth within 5-10 minutes. Sterilize forceps will be used for each carrier transfer. From each tube of neutralizer, two mL will be subcultured to a tube containing 20 mL 7H9 broth and 2 mL will be subcultured to a tube containing 20 mL Kirchner's medium. Each subculture tube will be shaken thoroughly and the sequence will be repeated for all carriers within 30 ± 5 minutes.

F. Controls:

1. Viability controls:

One contaminated carrier each will be added to tubes containing 20 mL MPB, 20 mL 7H9 and 20 mL KM. The tubes will be incubated with the test in order to confirm the ability of the recovery media to support growth of the challenge microorganism.

2. Neutralizer effectiveness:

One sterile carrier per lot will be exposed to test material and processed in the same manner as the test carriers. To each tube of 20 mL 7H9, 20 mL KM and 20 mL MPB media, fewer than 100 CFU per tube will be added.

The concentration of the bacterial suspension inoculated into these tubes will be confirmed by spread plating using 7H11 agar plates in duplicate and the plates will be incubated for 17-21 days at 36±1C. The tubes will be incubated with the test to confirm neutralization.

3. Small numbers control (Neutralizer toxicity):

To provide comparison for confirmation of growth in the neutralizer effectiveness control tubes, one uninoculated and untreated carrier will be transferred to a tube containing 10 mL of the Neutralizer and shaken thoroughly. The carrier will be transferred to one tube containing 20 mL of MPB. From the tube of Neutralizer, 2 mL will be transferred to a 20 mL tube

of 7H9 and 2 mL will be transferred to a 20 mL tube of KM. The subculture media (MPB, 7H9, and KM) will be inoculated with fewer than 100 CFU / tube. These tubes will be incubated with the test.

4. Sterility controls:

One sterile, uninoculated carrier will be placed into a tube of MPB broth. In addition, 1 tube of each subculture medium with 2 mL sterile neutralizer will be incubated for quality control purposes. Each tube will be shaken thoroughly and all the tubes will be incubated with the efficacy test. Duplicate 7H11 agar plates will also be incubated with the test.

5. Carrier counts:

Three inoculated dried carriers will be randomly selected for evaluation. One carrier will be assayed immediately prior to conducting the efficacy test and two carriers following the test. Each of three carriers will be placed in independent sterile 50 mL polypropylene conical tubes containing 20 mL of MPB and subjected to vortex for 15 seconds.

After vortex mixing for 15 seconds, each tube will be briefly mixed on a vortex mixture and 10- fold serial dilution will be conducted in PBDW. 100 µL aliquots of appropriate dilutions will be plated in duplicate on M7H11 using spread plating. Dilutions 10^{-1} through 10^{-3} should produce plates with CFU in the appropriate range. Plates must be dry prior to incubation. All dilutions and plating will be performed within 2 hours of vortexing. The plates will be incubated for 17-21 days at 36±1C.

6. Performance assessment of Media:

The standardized culture will be serially diluted 10-fold out to the 10^{-6}. For solid media, 0.1 mL aliquots from the 10^{-3} to the 10^{-6} dilutions will be spread plated on 7H11 agar plates in duplicate. The plates will be incubated for 17-21 days at 36±1C. Plate counts of 30-300 CFU/plate should result from at least one of the dilutions plated.

For liquid media, each tube of liquid medium (MPB, 7H9 and KM) will be inoculated with 0.1 mL aliquots from the 10^{-3} to the 10^{-6} dilutions in duplicate. Tubes will be incubated for 60 days at 36±1C. At least 1 of the 2 tubes in a set should show growth that received a low level of inoculum

G. Incubation:

Unless otherwise indicated, all test tubes used for secondary transfers (MPB, 7H9 and KM) and all controls tubes will be incubated for 60 days at 36±1C and the results will be reported as growth or no growth. If no test culture shows visible growth, the test will be incubated an additional 30 days before the final reading is made. All plates will be incubated for 17-21 days at 36±1C, the colonies will be counted and the average CFU calculated.

H. Confirmation of challenge microorganism:

On the day of the final reading, acid-fast stains will be performed for all test culture tubes demonstrating visible growth and two viability control tubes in order to verify the presence of the challenge microorganism. In addition, the culture morphology will be observed.

PRODUCT EVALUATION CRITERIA:

The test agent meets effectiveness requirements if no visible growth occurs in any replicate tube, for any of the subculture broths.

TEST ACCEPTANCE CRITERIA:

The test will be acceptable if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance. There are no proposed statistical methods for this test.

- The carrier counts must be at least 4 – 6 Log_{10} colony-forming units (CFU)/carrier. The log_{10} density (LD) for each carrier will be determined based on the following:
  - Dilutions yielding counts up to 300 CFU will be used.
  - Plate counts of 0 will be included in the calculations.
  - The CFU/mL (of broth) will be calculated:

$$\text{CFU/mL} = \frac{(\text{avg. CFU for } 10^{-x}) + (\text{avg. CFU for } 10^{-y}) + (\text{avg. CFU for } 10^{-z})}{10^{-x} + 10^{-y} + 10^{-z}}$$

  - The CFU/carrier will be calculated by multiplying the CFU/mL by the volume of broth into which the bacteria were harvested from the carrier by vortexing (20 mL).
  - The LD for each carrier will be calculated by taking the Log_{10} of the density (per carrier).
  - The mean LD across carriers is the mean Test LD for the test. The mean Test LD must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 6.0 (corresponding to a geometric mean density of 1.0×10^6).
- Following inoculation of <100 CFU per tube/plate, growth must be observed in the small numbers control and the performance assessment of Media.
- The sterility controls must show no growth.
- The viability controls must show growth.
- The neutralization confirmation tubes must show growth following inoculation with <100 CFU per tube to confirm effective neutralization.
- Neutralization Confirmation and Small Number Control must be 100 CFU/tube or less.

DATA PRESENTATION:

The final report will include the following information:

- The number of positive carriers.
- The average colony-forming units per carrier.
- The results of all controls.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned before initiation of the test. Resumes for technical personnel are maintained and are available on request. This study will be conducted at MicroBioTest, 105 Carpenter Drive, Sterling, VA 20164.

CONFIDENTIALITY:

All data generated at MicroBioTest are held in strictest confidence and are available only to the sponsor and the sponsor designated authorities (if applicable). In turn, no reference to MicroBioTest's promotion of the evaluated test articles may be made public by the sponsor.

REPORT FORMAT:

MicroBioTest employs a standard report format for each test design. Each final report provides the following information:

- Sponsor identification and test agent identification
- Type of test and project number
- Dates of study initiation and completion
- Interpretation of results and conclusions
- Test results
- Methods and evaluation criteria
- Signed Quality Assurance and Compliance Statements (if applicable)

REGULATORY COMPLIANCE AND QUALITY ASSURANCE (applicable to GLP studies only)

This study will be performed in compliance with the US Environmental Protection Agency's Good Laboratory Practices regulations, 40 CFR 160. Note: information on the identity, strength, purity, stability, uniformity, and dose solution analysis of the test agent resides with the sponsor of the study unless otherwise stated.

The Quality Assurance Unit of MicroBioTest will inspect the conduct of the study for GLP compliance. The dates of the inspections and the dates that findings are reported to the study management and study director will be included in the final report.

RECORDS TO BE MAINTAINED:

All raw data, protocol, protocol modifications, test agent records, final report, and correspondence between MicroBioTest and the sponsor will be stored in the archives at MicroBioTest, 105 Carpenter Drive, Sterling, Virginia 20164 or in a controlled facility off site.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test agent; challenge microorganism used; media and reagent identification; and the type of neutralizers employed in the test will be addressed in a project sheet issued separately for each study. The date the study director signs the protocol will be the initiation date. All project sheets will be forwarded to the study sponsor.

MISCELLANEOUS INFORMATION: The following information is to be completed by sponsor before initiation of study

A. Name and address: Professional Disposables International, Inc.
Two Nicepak Park
Orangeburg, NY 10962

B Test agent and test information:

Test agent name	Wonder Woman Formula-B Wipes	
Active ingredient(s)	0.55% Quat, 27% Ethanol, 27% IPA	
Lot No. Prepared at the Lower Certified Limit (LCL)	Lot 1	Lot 2
	7921-AE-758-191A	7921-AE-758-191B
Manufacture Date	12/03/14	12/03/14
LCL (Yes/No)	Yes – LCL	Yes – LCL

Note: the towelettes (wipes) will be supplied in individual packets (one wipe per package). Each packet will be numbered and will include the sponsor measure weight value. Each packet will be weighed by MicroBioTest just prior to testing to verify that the weight matches the sponsor measure weight within +/- 2% range. If it does not, the weight will be documented and the wipe will not be used.

C. Test conditions:

Contact time	ONE (1) minute(s)
Contact temperature	■ Ambient (20±1C)
Dilution	■ Not applicable (Towelette)
Organic load in inoculum	■ 5% Heat-inactivated Fetal Bovine Serum
Wiping Instruction	■ Refer to protocol page 6

D Precautions/storage conditions MSDS provided ■ yes ☐ no

REPORT HANDLING AND STUDY CONDUCT: Health Canada, EPA, GLP

PROTOCOL APPROVAL:

Sponsor Signature: _____ Date: 12/6/14
Phyllis Vitolo, Professional Disposables International, Inc.

Study Director Signature: _____ Date: 12/17/14
Shirshendu Saha, MicroBioTest